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PRESS

Biochemical and Biophysical Research Communications 293 (2002) 396–402

BBRC

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## Isolation and identification of EG-VEGF/prokineticins as cognate ligands for two orphan G-protein-coupled receptors<sup>☆</sup>

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Received 18 March 2002

### Abstract

Endocrine gland-derived vascular endothelial growth factor (EG-VEGF, identical to prokineticin 1) is a novel peptide recently identified as a selective mitogen for endocrine gland endothelial cells. The present study demonstrates that EG-VEGF/prokineticin 1 and a peptide closely related to EG-VEGF, prokineticin 2, are cognate ligands of two orphan G-protein-coupled receptors designated ZAQ (=EG-VEGF/PK-R1) and 15E (=EG-VEGF/PK-R2). EG-VEGF/prokineticin 1 and prokineticin 2 induced a transient increase in intracellular calcium ion concentration ( $[Ca^{2+}]_i$ ) with nanomolar potency in Chinese hamster ovary (CHO) cells expressing EG-VEGF/PK-R1 and -R2 and bind to these cells with high affinity and with different receptor selectivity. EG-VEGF/prokineticins provoke rapid phosphorylation of p44/42 MAP kinase and DNA synthesis in the bovine adrenal capillary endothelial cells (BACE). The mRNAs of both EG-VEGF/PK-R1 and -R2 were expressed in BACE. The identification of the receptors for EG-VEGF/prokineticins may provide a novel molecular basis for the regulation of angiogenesis in endocrine glands. © 2002 Elsevier Science (USA). All rights reserved.

**Keywords:** G-protein-coupled receptor; Prokineticin; EG-VEGF; MIT1; GPR73; EG-VEGF/PK-R; Bovine adrenal capillary endothelial cells; MAP kinase; Thymidine incorporation.

A wide variety of biologically active substances exert their activity by binding to G-protein-coupled receptors (GPCRs). Recent progress in genome DNA research has identified a large number of genes that encode GPCRs. Many of these are called orphan GPCRs, for which the

cognate ligands remain to be unknown. Orphan GPCRs have been used to discover their novel endogenous peptide ligands such as nociceptin/orphanin FQ [1,2], prolactin-releasing peptide [3], orexins [4], apelin [5], ghrelin [6], and metastatin [7].

EG-VEGF is a novel angiogenic mitogen that is selective for endocrine gland endothelial cells, and which was recently identified among a library of secreted proteins [8]. EG-VEGF is identical to prokineticin 1 [9], which was recently cloned as a mammalian homolog of mamba intestinal toxin-1 (MIT1) [10]. Prokineticin 1 has a family peptide, prokineticin 2 [9], which is also known as a mammalian homolog of frog skin peptide Bv8 [11]. EG-VEGF/prokineticin 1, prokineticin 2, MIT1, and Bv8 [12] contain 10 cysteine residues in their molecules that are in identical positions, suggesting that these peptides have a common evolutionary origin. MIT1 and prokineticins have been

<sup>☆</sup>The nucleotide sequence reported in this paper has been submitted to GenBank under Accession Nos. AY089983, AY089984, AY089972, AY089973, AY089974, AY089975, and AY089976.

**Abbreviations:** GPCR, G-protein-coupled receptor; EG-VEGF, endocrine gland vascular endothelial growth factor; CHO, Chinese hamster ovary; BACE, bovine adrenal capillary endothelial cells; RACE, rapid amplification of cDNA end; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-tetramethylrhodamine; FBS, fetal bovine serum; FLIPR, fluorometric imaging plate reader; EC<sub>50</sub>, half maximum effective concentration; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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reported to contract gastrointestinal smooth muscles [9,13], although the molecular structure of the receptor remained undefined.

We now report the isolation EG-VEGF/prokineticin 1 from bovine milk and the identification of EG-VEGF/prokineticins as cognate ligands for two closely related orphan GPCRs, ZAQ (=EG-VEGF/PK-R1), and I5E (=EG-VEGF/PK-R2). In addition, we report the tissue distribution of rat EG-VEGF/prokineticins and their receptors' mRNAs and identify the mRNAs of the two receptors in BACE. The identification of EG-VEGF/prokineticin receptors should facilitate the development of novel therapeutics for diseases involving excessive angiogenesis in the endocrine glands.

## Materials and methods

**Cloning of ZAQ and I5E cDNAs and their expression in CHO cells.** Two regions of human ZAQ sequence showing high similarity to human I5E (Patent no. WO9846620) were found in GenBank Database (accession number Z69648 and AQ419390). The 5'- and 3'-ends of the sequence were verified by 5'- and 3'-rapid amplification of cDNA ends (RACE) and full-length human ZAQ cDNA (AY089976) was cloned by PCR with primers 5'-GTCGACATGGAGACCACCATGGGGTTCATGG-3' and 5'-ACTAGTTTATTTTAGTCTGATGCAGTCCACCTCTTC-3'. Full-length human I5E cDNA was cloned by PCR using gene specific primers. The cDNAs obtained were introduced into an expression plasmid, pAKKO-111H [14] and stably expressed in CHO dhFr-cells by a previously described method [15].

Partial fragment of rat ZAQ cDNA was isolated from rat brain Marathon ready cDNA library (Clontech) by PCR using primers, 5'-GTGGTRCGSCAGCTCTCTGGGAGCA-3' and 5'-CATGCTGTTGCTCATGGCGATGCACTC-3'. The 5'- and 3'-ends of the fragment were extended by 5'- and 3'-RACE using rat brain Marathon ready cDNA library to obtain the full-length cDNA (AY089974). Rat I5E cDNA clone (AY089975) was obtained from rat brain SuperScript Rat cDNA library (Gibco BRL) using Gene Trapper cDNA Positive Clone Selection System (Life Technologies) with biotinylated probes, 5'-CCTCACCAAYCTGCTYATYGCCAACCTGGCC-3' and 5'-GTGGTRCGSAGCTCTCTGGGAGCA-3'.

Partial fragments of bovine ZAQ and I5E cDNAs were obtained from BACE by PCR using degenerate primers, 5'-ATYGTSTGCTGCCCCTYAGATG-3' and 5'-TAGTAGAGCTGCTGRTCCACBGRCC-3'. The 5'- and 3'-ends of the fragment were extended by 5'- and 3'-RACE and the full-length coding sequences (AY089972 and AY089973) were obtained by PCR using primers, 5'-GCTCCTCCCGTTCTTTGAAATC-3' and 5'-TGTAAGTCTCTGAATCTCATGTTGGCC-3' for ZAQ, 5'-GCTGGGTGAGAAGGAATAGGGA-3' and 5'-TGCTCTTAATCTCGCTGGTGGT-3' for I5E.

**Purification of the cognate ligand for ZAQ.** Three liters of bovine milk was centrifuged and resulting supernatant was acidified with 1 M acetic acid and centrifuged. The supernatant was mixed with two volumes of acetone for protein-precipitation, after centrifugation to remove the precipitate, the clear supernatant was extracted with diethyl ether. The aqueous phase was evaporated and then loaded onto a  $C_{18}$  reversed-phase column (Prep  $C_{18}$ , Waters) and eluted with 60%  $CH_3CN/0.1\%$  TFA. The eluate lyophilized was dissolved in 20 mM  $HCOONH_4$  (pH 4)—25%  $CH_3CN$  and loaded onto an SP-Sephadex C-25 (Amersham Pharmacia Biotech). The column was successively eluted with 200 mM, 500 mM, and 1000 mM  $HCOONH_4$  containing 25%  $CH_3CN$ . The 1000-mM eluate was lyophilized and separated by reverse-phase column (TSKgel ODS-80T's, 4.6 × 250 mm, Tosoh) with

a linear gradient of 20–60%  $CH_3CN$  in 0.1% TFA for 80 min at 1 ml min<sup>-1</sup>. The active fractions were further fractionated by reverse-phase column (TSKgel Super-Phenyl, 4.6 × 100 mm, Tosoh) with a linear gradient of 15–40%  $CH_3CN$  in 0.1% TFA for 75 min at 1 ml min<sup>-1</sup> and finally purified by reverse-phase column ( $\mu$ RPC C2/C18 ST 4.6/100, 4.6 × 100 mm, Amersham Pharmacia Biotech) with a linear gradient of 35–50%  $CH_3CN$  in 0.1% heptafluorobutyric acid for 60 min at 1 ml min<sup>-1</sup>. The amino acid sequence of the peptide purified was analyzed with a protein sequencer (PE Biosystems Procise 491cLC).

**Calcium-mobilization assay using a fluorometric imaging plate reader (FLIPR).** The receptor-expressing cells were seeded (30,000 cells/well) into 96-well black-wall microplates 16–24 h before assay. The cells were incubated for 1 h at 37 °C with 4  $\mu$ M Fluo-3 AM (Dojindo) in H/HBSS (Hanks' balanced salts solutions supplemented with 20 mM HEPES, pH 7.4) containing 2.5 mM probenecid and 0.1% fetal bovine serum (FBS), and then washed four times with the assay buffer. Changes in  $[Ca^{2+}]_i$  were measured using a FLIPR (Molecular Devices).

**Purification of MIT1 from black mamba venom.** MIT1 was purified from black mamba venom (Sigma) monitoring agonist activity for ZAQ using a FLIPR. The venom was fractionated by reverse-phase column (Wakosil-II 5C18HG Prep, 20 × 250 mm, Wako) with a linear gradient of 20–40%  $CH_3CN$  in 0.1% TFA for 120 min at 5 ml min<sup>-1</sup>. The active fractions were lyophilized and then purified by cation exchange column (TSKgel CM-2SW, 4.6 × 250 mm, Tosoh) with a linear gradient of 10–1000 mM  $HCOONH_4$  (pH 6.6) containing 25%  $CH_3CN$  for 90 min at 1 ml min<sup>-1</sup>. The peptide was finally purified by reverse-phase column (Vydac 218 TP510, 4.6 × 100 mm, Vydac) with a linear gradient of 15–35%  $CH_3CN$  in 0.1% TFA for 75 min at 1 ml min<sup>-1</sup>.

**Cloning of EG-VEGF/prokineticin 1 and prokineticin 2 cDNAs.** Partial fragment of rat EG-VEGF/prokineticin 1 cDNA was cloned from rat brain cDNA library by degenerate PCR with primers, 5'-TCA CCYCAAGTGAYCATGAGAGG-3' and 5'-CTAAAARTTGTRYRTTCTTCAAGTCC-3' for the first PCR, and 5'-ATCACAGGGGCCTGTGARCG-3' and 5'-AGCAGCGGTACCTGCCGTCC-3' for nested PCR. After 5'- and 3'-RACE for extending the external sequences, the full-length cDNA of rat EG-VEGF/prokineticin 1 (AF089983) was isolated by nested PCR with primers 5'-GATCATGAGAGGTGCTGTGCAAGTCTTC-3' and 5'-CAGATGTAACACAAGAGGTCAACCCAGTAGG-3' following the first PCR with primers 5'-ATTCCAGAGTGGACAGTGTTCCTTCACC-3' and 5'-CTCTCTGCACGCTGCTGGACTGTTCC-3'. Partial fragment of rat prokineticin 2 cDNA was obtained by degenerate PCR using rat testis Marathon ready cDNA library and following primers: 5'-GCTTGYGACAAGGACTCYCA-3' and 5'-GTTYCTACTYAGAGYGAT-3'. After 5'- and 3'-RACE for extending the external sequence, the full-length cDNA of rat prokineticin 2 (AY089984) was isolated from rat brain cDNA by nested PCR with primers, 5'-GGGACGCCATGGAGGAC-3' and 5'-TTTCCAGCTCCTGCTTCAGA-3' following the first PCR with primers 5'-TAACCGCCACCGCCTCCT-3' and 5'-CGAGACTTGACAGACATTGTTCAAGT-3'.

**Expression of human EG-VEGF/prokineticin 1 and prokineticin 2 in Escherichia coli.** The double-stranded DNA encoding human EG-VEGF/prokineticin 1 or prokineticin 2 was obtained by annealing six synthetic oligonucleotides, and inserted into the *Nde*I–*Bam*HI cloning site of the pTCII vector [16]. The expression plasmid was introduced into *E. coli* MM294 (DE3) and recombinant human EG-VEGF/prokineticin 1 and prokineticin 2 were produced under the control of T7 promoter. Recombinant EG-VEGF/prokineticin 1 or prokineticin 2 was extracted from *E. coli* cells with extraction buffer (7 M guanidine-HCl, 200 mM Tris, pH 8.0), refolded with refolding buffer (50 mM Tris, 0.4 M L-arginine, 1 mM reduced glutathione, and 0.2 mM oxidized glutathione, pH 8.5), and purified using a TSKgel CM-5PW column (21.5 × 150 mm, Tosoh) and C4P-50 column (21.5 × 300 mm, Showa Denko). The purified peptide was proved to be homogeneous on SDS-PAGE and analytical HPLC.

**Receptor binding assay.** The assay was performed by a previously described method [15] with minor modification. MIT1 was radiolabeled with [<sup>125</sup>I]Bolton–Hunter reagent (NEX120, NEN) and the peptide radiolabeled ([<sup>125</sup>I]BH-MIT1) was purified using reverse-phase column (TSKgel Super-ODS, 4.6 × 100 mm, Tosoh). The cells were inoculated into 24-well plates and cultured for 2 days. The cells were washed with assay buffer (H/HBSS containing 0.2% bovine serum albumin (BSA)) and were incubated with 100 pM [<sup>125</sup>I]BH-MIT1 and peptide samples for 37 °C for 1 h. The cells were then washed with assay buffer and then solubilized with 0.5 N NaOH/0.1% SDS. Radioactivity was measured with a γ-ray counter. Nonspecific binding was determined in the presence of 1 μM unlabeled MIT1.

**Detection of p44/42 MAP kinase activation.** BACE were obtained as described by Folkman et al. [17]. BACE were plated into 12-well plates and cultured for 1 day. After BACE were treated with peptide samples for 5 min at 37 °C, the supernatant was removed and the cells were lysed with lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 50 mM NaF, 4 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 10 μg/ml pepstatin A, pH 7.4). The lysates were centrifuged and the supernatants were subjected to Western blot analysis using Phospho-p44/42 MAP Kinase (Thr 202/Tyr 204) Antibody (Cell Signaling Technology) and ECL-plus detection system (Amersham Pharmacia Biotech).

**Measurement of [<sup>3</sup>H]thymidine incorporation into BACE.** BACE were plated into 48-well plates and cultured for 1 day. After the cells were cultured with ligands in DMEM containing 0.1% FBS and 0.5% BSA for 16–20 h, the cells were labeled with [<sup>3</sup>H]thymidine (0.5 μCi/well) for another 8 h. The cells were then washed with cold H/HBSS and methanol and incubated with 10% trichloroacetic acid at 4 °C for 15 min. The acid-insoluble fraction was dissolved with 0.3 N NaOH and the radioactivity was counted using a scintillation counter.

**Real time PCR analysis for EG-VEGF/prokineticins and EG-VEGF PKRs mRNAs.** Total RNAs were prepared from multiple tissues of 7–8 week-old Wistar rats using Trizol reagent (Gibco) and poly(A)<sup>+</sup> RNAs were purified using mRNA purification kit (Amersham Pharmacia Biotech). cDNAs were synthesized using SuperScript Preamplification System for First Strand cDNA Synthesis (Life Technologies). Real time PCR analysis was carried out using a Prism 7700 Sequence Detector (PE Biosystems). For BACE, total RNA was prepared using RNeasy Mini kit (Qiagen) and was directly subjected to cDNA synthesis and real time PCR analysis. Specific primers and fluorescence-labeled probes used were: 5'-TTGCGAAGCTTTTGTAGCG-3', 5'-CAACTTCATCTT CATCATCGCTGGC-3', and 5'-Fam-CAACTTCATCTTTCATCA CTGCGCTGGC-3' for rat EG-VEGF/PK-R1; 5'-CAGACCTTCT TTGACGCCA-3', 5'-CAGACTAGCATGATGCC-3', and 5'-Fam-A ATCGTCATTGGCGTAGCCCTGGC-Tamra-3' for rat EG-VEGF/PK-R2; 5'-GTGCAAGTCTTCATCATGCTCCT-3', 5'-AGGGGCT GTGAACGAGAT-3', and 5'-Fam-CTAGCAACTGTCTCTGACT GTGCGGTGATC-Tamra-3' for rat EG-VEGF/prokineticin 1; 5'-CCCCCTGACTCGGAAAGTTC-3', 5'-CCAGGTTTGGCATGT TT AAGG-3', and 5'-Fam-AGGATGCACCACACTTGTCCCTGCCT-Tamra-3' for rat prokineticin 2; AACCAACTATTTCCCTCTGCTT GAC-3', 5'-TCACCGTAGCTGAAGTTGAAGG-3', and 5'-Fam-CCTC GGAGCCCAAGCTGCTTCTTT-Tamra-3' for bovine EG-VEGF/PK-R1; or 5'-TCATGAGAGCAGAAAGTCTGGA-3', 5'-TGGCTG GAAACTAGCATTTCC-3', and 5'-Fam-CACACACCGCTCACT GGAAAGCTTCA-Tamra-3' for bovine EG-VEGF/PK-R2.

## Results and discussion

During our attempts to search human genomic DNA database for novel GPCR genes, we found two DNA fragments of a putative GPCR gene. Based on these

sequences, we isolated the full-length cDNA encoding an orphan GPCR originally termed ZAQ. ZAQ showed significant homology to mouse GPR73 [18] and human orphan GPCR, 15E (Patent no. WO9846620, 90% identity, Fig. 1). It is thus suggested that ZAQ and 15E are the receptor subtypes for structurally related ligands. We subsequently isolated the rat and bovine ZAQ and 15E cDNAs. The deduced amino acid sequences of these proteins are shown in Fig. 1.

To identify the unknown cognate ligand for ZAQ, we monitored the agonist activity of the increase in [Ca<sup>2+</sup>]<sub>i</sub> in CHO cells stably expressing ZAQ using a FLIPR. ZAQ is structurally similar to neuropeptide Y receptors, but did not respond to known neuropeptides, including neuropeptide Y, peptide YY, and pancreatic polypeptide up to a concentration of 1 μM. During screening against several kinds of tissue extracts, we found that bovine

hZAO	MSITMGFMDDNATNTSTSLFSLVLPNGAHATSPFFNFSSDYDMPLEDEE	50
rZAO	MSITMGFMDDNATNTSTSLFSLVLPNGAHATSPFFNFSSDYDMPLEDEE	50
bZAO	MSITMGFMDDNATNTSTSLFSLVLPNGAHATSPFFNFSSDYDMPLEDEE	50
h15E	MAAQNGNTSFTNPNPFPQCHASSLSFNFSYGDYDMPLEDEE	41
r15E	MGCQNGNTSFPAPLNPFPQCHASSLSFNFSYGDYDMPLEDEE	41
b15E	MAAQNGNTSFPAPLNPFPQCHASSLSFNFSYGDYDMPLEDEE	41
TM1		
hZAO	QVTSNRTFPAAKIVIGMALVQIMVCGICNFFFLAALRYKKRLNLTNLL	100
rZAO	QVTSNRTFPAAKIVIGMALVQIMVCGICNFFFLAALRYKKRLNLTNLL	100
bZAO	QVTSNRTFPAAKIVIGMALVQIMVCGICNFFFLAALRYKKRLNLTNLL	100
h15E	DMTKTRTPPAKIVIGMALVQIMVCGICNFFFLAALRYKKRLNLTNLL	91
r15E	DMTKTRTPPAKIVIGMALVQIMVCGICNFFFLAALRYKKRLNLTNLL	91
b15E	DMTKTRTPPAKIVIGMALVQIMVCGICNFFFLAALRYKKRLNLTNLL	91
TM2		
hZAO	IANLAIADPLVAIVCCPFENDITYVROLSEWEGHVLCSVNYLRTVSLV	150
rZAO	IANLAIADPLVAIVCCPFENDITYVROLSEWEGHVLCSVNYLRTVSLV	150
bZAO	IANLAIADPLVAIVCCPFENDITYVROLSEWEGHVLCSVNYLRTVSLV	150
h15E	IANLAIADPLVAIVCCPFENDITYVROLSEWEGHVLCSVNYLRTVSLV	142
r15E	IANLAIADPLVAIVCCPFENDITYVROLSEWEGHVLCSVNYLRTVSLV	141
b15E	IANLAIADPLVAIVCCPFENDITYVROLSEWEGHVLCSVNYLRTVSLV	141
TM3		
hZAO	STNALLAIAIDRYLAIVHPLKPRMYOTAFLLALVMVSTLIATPSAFL	200
rZAO	STNALLAIAIDRYLAIVHPLKPRMYOTAFLLALVMVSTLIATPSAFL	200
bZAO	STNALLAIAIDRYLAIVHPLKPRMYOTAFLLALVMVSTLIATPSAFL	200
h15E	STNALLAIAIDRYLAIVHPLKPRMYOTAFLLALVMVSTLIATPSAFL	191
r15E	STNALLAIAIDRYLAIVHPLKPRMYOTAFLLALVMVSTLIATPSAFL	190
b15E	STNALLAIAIDRYLAIVHPLKPRMYOTAFLLALVMVSTLIATPSAFL	191
TM5		
hZAO	TTFTVLIVKSOEKLFCGQWVPVDOOYLYKSYFLFPGIEFVGPIVMTL	250
rZAO	TTFTVLIVKSOEKLFCGQWVPVDOOYLYKSYFLFPGIEFVGPIVMTL	250
bZAO	TTFTVLIVKSOEKLFCGQWVPVDOOYLYKSYFLFPGIEFVGPIVMTL	250
h15E	ATFTVLIVKSOEKLFCGQWVPVDOOYLYKSYFLFPGIEFVGPIVMTL	241
r15E	TTFTVLIVKSOEKLFCGQWVPVDOOYLYKSYFLFPGIEFVGPIVMTL	240
b15E	TTFTVLIVKSOEKLFCGQWVPVDOOYLYKSYFLFPGIEFVGPIVMTL	241
TM6		
hZAO	CYARISRELNPKAVGPGFQTEQIRKELCRKKTIVLLMCLTAYVLCNAPF	300
rZAO	CYARISRELNPKAVGPGFQTEQIRKELCRKKTIVLLMCLTAYVLCNAPF	300
bZAO	CYARISRELNPKAVGPGFQTEQIRKELCRKKTIVLLMCLTAYVLCNAPF	300
h15E	CYARISRELNPKAVGPGFQTEQIRKELCRKKTIVLLMCLTAYVLCNAPF	291
r15E	CYARISRELNPKAVGPGFQTEQIRKELCRKKTIVLLMCLTAYVLCNAPF	290
b15E	CYARISRELNPKAVGPGFQTEQIRKELCRKKTIVLLMCLTAYVLCNAPF	291
TM7		
hZAO	YGFTIVRDPFPTVVKCKHYLTAFVVECIAMNSMINTVCPVTQKNTM	350
rZAO	YGFTIVRDPFPTVVKCKHYLTAFVVECIAMNSMINTVCPVTQKNTM	350
bZAO	YGFTIVRDPFPTVVKCKHYLTAFVVECIAMNSMINTVCPVTQKNTM	350
h15E	YGFTIVRDPFPTVVKCKHYLTAFVVECIAMNSMINTVCPVTQKNTM	341
r15E	YGFTIVRDPFPTVVKCKHYLTAFVVECIAMNSMINTVCPVTQKNTM	340
b15E	YGFTIVRDPFPTVVKCKHYLTAFVVECIAMNSMINTVCPVTQKNTM	341
TM8		
hZAO	KYFKKMLLHWKSYNGKSSADLLKTKCPATBEVDCIRLK	393
rZAO	KYFKKMLLHWKSYNGKSSADLLKTKCPATBEVDCIRLK	393
bZAO	KYFKKMLLHWKSYNGKSSADLLKTKCPATBEVDCIRLK	393
h15E	KYFKKMLLHWKPSORGKSSADLLKTKCPATBEVDCIRLK	384
r15E	KYFKKMLLHWKPSHYGKSSADLLKTKCPATBEVDCIRLK	383
b15E	KYFKKMLLHWKPSHYGKSSADLLKTKCPATBEVDCIRLK	384

Fig. 1. Alignment of human, rat, and bovine ZAQ (EG-VEGF/PK-R1) and 15E (EG-VEGF/PK-R2) amino acid sequences. Shading denotes amino acid identity. Seven putative transmembrane domains (TM1–7) are indicated.

milk exhibited a strong activity that induced a robust and transient increase in  $[Ca^{2+}]_i$  (Fig. 2A). This activity was specific for ZAQ, since it failed to evoke an increase in

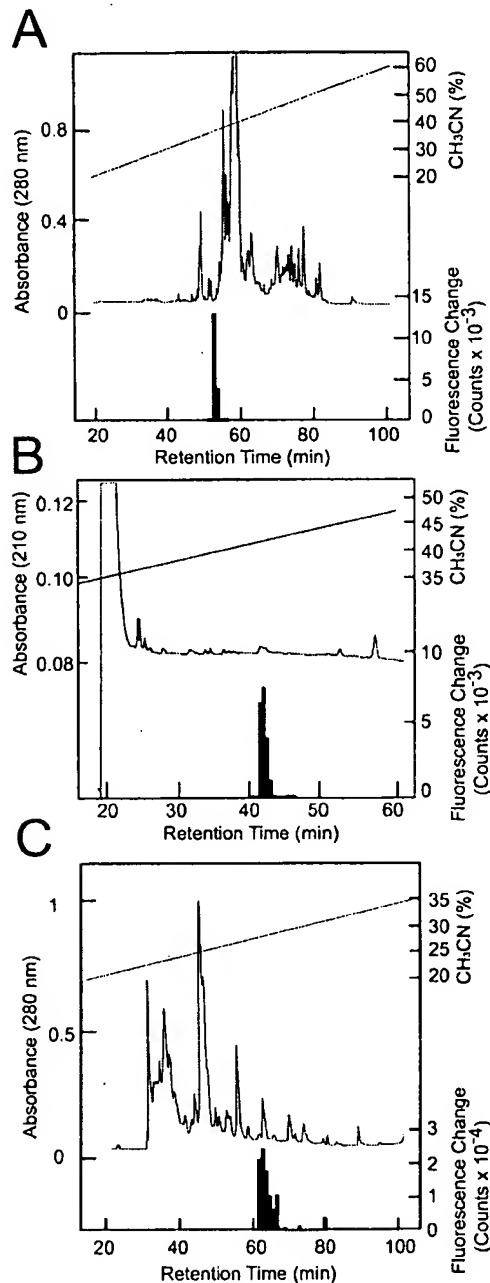


Fig. 2. Purification of EG-VEGF/prokineticin 1 from bovine milk and identification of MIT1 in black mamba venom. (A) The first HPLC purification step of EG-VEGF/prokineticin 1 from bovine milk by reverse-phase column (TSKgel ODS-80Ts). The specific fluorescence changes in EG-VEGF/PK-R1-expressing CHO cells were shown with black bars. (B) The final purification step of EG-VEGF/prokineticin 1 by reverse-phase column ( $\mu$ RPC C2/C18 ST 4.6/100). Active substance, corresponding to the absorbance peak, was subjected to a protein sequencer. (C) Identification of EG-VEGF/PK-R1-activating activity in black mamba venom. The venom was fractionated using reverse-phase column (Wakosil-II 5C18HG). Bars show specific fluorescence change in CHO cells expressing EG-VEGF/PK-R1.

$[Ca^{2+}]_i$  in control of CHO cells expressing  $ET_A$  receptor [15]. We purified the activity by successive steps of HPLC from bovine milk (Fig. 2B). The partial N-terminal amino acid sequence of the peptide isolated was determined to be AVITGAXERDVQXRAGTXXXAVSL (X, not identified). This sequence was remarkably similar to the N-terminal amino acid sequence of MIT1 [10] isolated from the black mamba (*Dendroaspis polylepis*) venom. A database search revealed the presence of a human expressed sequence tag (X40467). Since the clone was found to be partial, we performed a 3'RACE reaction with human testis cDNA as a template to obtain the full-length cDNA. After the completion of the cDNA cloning, the same sequence was reported as EG-VEGF [8] and prokineticin 1 [9] by two groups. EG-VEGF was identified among a library of human secreted proteins as a mitogen selective for endothelial cells derived from endocrine glands. Prokineticin 1 was cloned as a mammalian homolog of MIT1 with potent contractile activities for gastrointestinal smooth muscles. EG-VEGF/prokineticin 1 shares 80% identity with MIT1 and 58% identity with prokineticin 2 [9] (Fig. 3). Prokineticin 2 was also recently cloned as a mammalian homolog of the frog skin peptide, Bv8 [11]. We further isolated rat EG-VEGF/prokineticin 1 and prokineticin 2 cDNAs and found that these peptides are highly conserved between human and rat (93% and 95% similarity in mature form, respectively) (Fig. 3).

To confirm whether the MIT1-like molecule is cognate ligand for ZAQ, we examined the ZAQ-agonist activity of MIT1 and of recombinant EG-VEGF/prokineticin 1 and prokineticin 2. First, we examined the activity in black mamba venom and found that it showed a specific and an extremely strong activity (Fig. 2C). We then analyzed the purified active substance by mass spectrometry, sequenced it by Edman degradation,

hPK1	MRGATRVSIMLLLVTSVDCAVITGACERDVQCSAGTCCATSL	42
rPK1	MRGAVQVFIIMLLLATVSDCAVITGACERDVQCSAGTCCATSL	42
hPK2	MRSLCCAPLLLLLLLPPLLLTPRAGDAAVITGACDKDSQCSGGMCCAVSI	50
rPK2	MEDPRCAPLLLLLLLP-LLLLTPRAGDAAVITGACDKDSQCSGGMCCAVSI	50
MIT1	AVITGACERDLQCSKGTCCAVSL	23
Bv8	MKCPAQIVVLLLVIAFSGHAVITGACDKDVQCSGGTCCAVASA	42
hPK1	WLRLGLRMCTPLGREGECHPGSHKVPFFRKRKHHTCPCLPNLLCRFPDGG	92
rPK1	WLRLGLRLCTPLGREGECHPGSHKIPFFRKRQHHTCPSPSLLCRFPDGG	92
hPK2	WVKSLIRICTPMGKLGDSCHPLTRKVPFFGRMRHHTCPCLPGLACLRTPFN	100
rPK2	WVKSLIRICTPMGQVGDSCCHPLTRKVPFFGRMRHHTCPCLPGLACLRTPFN	100
MIT1	WIKSVRVCTPVGTSGEDCHPASHKIPPSGQRMHHTCPAPNLACVQTSFK	73
Bv8	WERNIRFCTPLGNSGEGCHPASHKVPYDGKRLSSLCPSKGLTCSK-SGE	91
hPK1	RYRCSMDLKNINF	105
rPK1	RYRCSQDLKIVNF	105
hPK2	RFICLAQR	108
rPK2	RFICLARK	108
MIT1	KPKCLSKS	81
Bv8	KPKCS	96

Fig. 3. Alignment of amino acid sequences of rat and human EG-VEGF/prokineticin 1 (PK1), prokineticin 2 (PK2), MIT1, and Bv8. Conservative cysteine residues are shown in boxes. Arrowhead indicates the cleavage site of signal peptide.

and identified it as MIT1 (C-terminal serine residue deleted).

We next produced recombinant human EG-VEGF/prokineticin 1 and prokineticin 2 in *E. coli* and CHO cells. We examined the agonist activity of the peptides for ZAQ and the presumed ZAQ subtype, I5E, using a FLIPR. Since the recombinant EG-VEGF/prokineticin 1 and prokineticin 2 produced in *E. coli* and CHO cells showed comparable agonist activity for the two receptors, we used the recombinant peptides produced in *E. coli* in the following experiments. EG-VEGF/prokineticin 1 and prokineticin 2 induced a dose-dependent transient increase in  $[Ca^{2+}]_i$  in ZAQ- and I5E-expressing CHO cells (Fig. 4A and B). The half maximum effective concentration ( $EC_{50}$ ) of EG-VEGF/prokineticin 1 and prokineticin 2 was  $140 \pm 29$  and  $15 \pm 2$  pM, for ZAQ and  $2900 \pm 530$  and  $150 \pm 31$  pM for I5E, respectively ( $n = 5$ ). These values are low enough to make EG-VEGF/prokineticin 1 and prokineticin 2 good candidates for cognate ligands for ZAQ and I5E. It is interesting that non-mammalian homolog of EG-VEGF/prokineticins, MIT1, showed the most potent agonist activity for ZAQ and I5E with  $EC_{50}$  values of  $13 \pm 2.1$  and  $34 \pm 11$  pM, respectively ( $n = 5$ ) (Fig. 4A and B).

Radioligand-binding studies were then performed to further characterize these ligands/receptors pairing. The binding of  $[^{125}I]$ BH-MIT1 to ZAQ- and I5E-expressing CHO cells was inhibited by a nanomolar concentration of

unlabeled prokineticins and MIT1 in a dose-dependent manner (Fig. 4C and D). The concentration of unlabeled ligands to inhibit 50% of specific radioligand binding ( $IC_{50}$ ) for EG-VEGF/prokineticin 1, prokineticin 2, and MIT1 was  $250 \pm 31$ ,  $6.9 \pm 1.2$ , and  $4.1 \pm 0.52$  nM for ZAQ,  $81 \pm 7.4$ ,  $7.6 \pm 0.90$ , and  $0.67 \pm 0.21$  nM for I5E, respectively ( $n = 4$ ). These results from the functional and binding assay confirm that EG-VEGF/prokineticin 1 and prokineticin 2 are cognate ligands for both ZAQ and I5E, which we now designate EG-VEGF/PK-R1 and EG-VEGF/PK-R2, respectively. It was shown that EG-VEGF/prokineticin 1 and prokineticin 2 shared EG-VEGF/PK-R1 and -R2, although the two receptors showed different ligand selectivity. EG-VEGF/PK-R1 is a MIT1- and prokineticin 2-preferable receptor, while EG-VEGF/PK-R2 is a MIT1-selective receptor.

Since it was demonstrated that EG-VEGF shows mitogenic activity for BACE [8], we examined the effects of prokineticin 2 and MIT1 on mitogenic parameters for BACE. First, we determined whether EG-VEGF/prokineticin 1, prokineticin 2, and MIT1 activated p44/42 MAP kinase (MAPK) in BACE. Several lines of evidence have shown that MAPK are important integrators for cell proliferation [19]. Activation of p44/42 MAPK was detected by Western blot analysis with Phospho-p44/42 MAP Kinase (Thr 202/Tyr 204) Antibody that specifically recognizes the activated form of p44/42 MAPK. EG-VEGF/prokineticin 1, prokineticin 2, and MIT1 stimulated dose-dependent phosphorylation of p44/42 MAPK with a rank order of potency as follows: MIT1 > prokineticin 2 > EG-VEGF/prokineticin 1 (Fig. 5A). Furthermore these three peptides promoted  $[^3H]$ thymidine incorporation into DNA with similar ligand selectivity (Fig. 5B). The  $EC_{50}$  values of EG-VEGF/prokineticin 1, prokineticin 2, and MIT1 for this activity were  $1400 \pm 240$ ,  $66 \pm 14$ , and  $22 \pm 5.9$  pM, respectively ( $n = 5$ ). These three peptides showed strong similarity in both structure and mitogenic activity for capillary endothelial cells derived from the endocrine gland.

To investigate the putative physiological roles of the novel ligands/receptors family, we examined the tissue distribution of EG-VEGF/prokineticin 1 and prokineticin 2 and their receptors in rat using a real time PCR analysis (Fig. 6A and B). The expression of EG-VEGF/prokineticin 1 was generally lower than that of prokineticin 2 in the tissues examined. The expression of prokineticin 2 was highest in the testis. Moderate level of expression was seen in the cerebrum, thymus, lung, spleen, ovary, and skeletal muscle.

To compare the localization of the two peptides with their receptors, we also examined the expression patterns of EG-VEGF/PK-R1 and -R2 in the same set of rat tissues. The result showed distinct expression patterns for the two receptors. EG-VEGF/PK-R1 mRNA was widely distributed in peripheral tissues with the highest level in the spleen and moderate levels in the

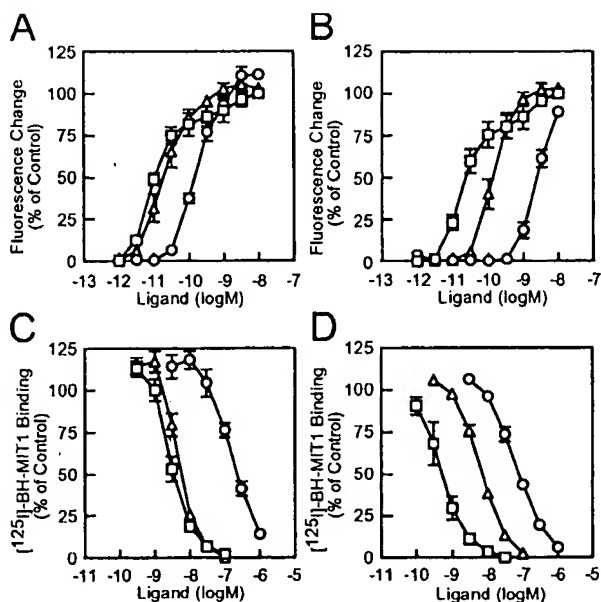


Fig. 4. Pharmacological characterization of EG-VEGF/PK-Rs. (A,B) Dose-response relationship of the increase in  $[Ca^{2+}]_i$  by EG-VEGF/prokineticin 1 (circle), prokineticin 2 (triangle), and MIT1 (square) in CHO cells expressing EG-VEGF/PK-R1 (A) or -R2 (B). (C,D) Competitive radioligand binding assays with CHO cells expressing EG-VEGF/PK-R1 (C) or -R2 (D). Symbols used are the same as in A and B. Data are means  $\pm$  SE of 4–5 individual experiments.

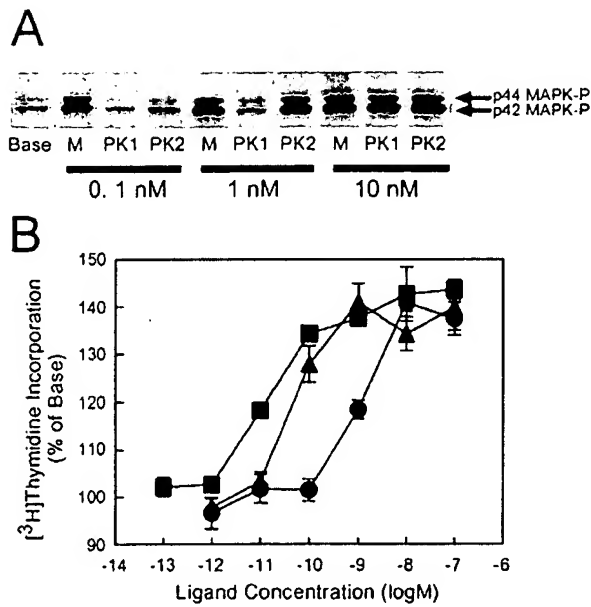


Fig. 5. Effects of EG-VEGF/prokineticin 1, prokineticin 2, and MIT1 on p44/42 MAPK phosphorylation and DNA synthesis in BACE. (A) phosphorylation of p44/42 MAPKs induced by indicated concentration of EG-VEGF/prokineticin 1 (PK1), prokineticin 2 (PK2), and MIT1 (M) was detected with Phospho-p44/42 MAP Kinase (Thr 202/Tyr 204) Antibody. (B) [ $^3\text{H}$ ]thymidine incorporation induced by EG-VEGF/prokineticin 1 (circle), prokineticin 2 (triangle), and MIT1 (square). Data are mean  $\pm$  SE of five individual experiments.

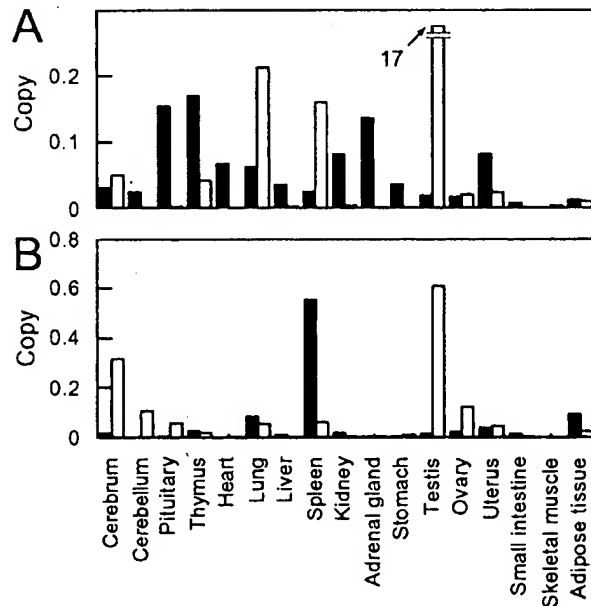


Fig. 6. Tissue distributions of EG-VEGF/prokineticins and their receptors mRNAs in rats analyzed quantitative RT-PCR analyses. (A) EG-VEGF/prokineticin 1, black bars; prokineticin 2, white bars. (B) EG-VEGF/PK-R1, black bars; EG-VEGF/PK-R2, white bars. Values are expressed as ratio to GAPDH  $\times$  100 for prokineticin 2, EG-VEGF/PK-R1 and -R2, and as ratio to GAPDH  $\times$  1,000 for EG-VEGF/prokineticin 1.

adipose tissues, thymus, lung, kidney, testis, uterus, and small intestine. In contrast, EG-VEGF/PK-R2 mRNA was expressed abundantly in the central nervous system and reproductive organs with the highest levels in the cerebrum, cerebellum, testis, and ovary. To examine the receptor subtype expressed in BACE, we cloned bovine EG-VEGF/PK-R1 and -R2 and quantified the mRNAs of the two receptors. The mRNAs of both EG-VEGF/PK-R1 and -R2 were expressed in BACE (82 copies/ng total RNA for EG-VEGF/PK-R1 and 77 copies/ng total RNA for EG-VEGF/PK-R2), indicating that the mitogenic activity of EG-VEGF/prokineticin 1 and prokineticin 2 is mediated by EG-VEGF/PK-R1 and/or EG-VEGF/PK-R2. Further studies are required to examine which receptor subtype is primarily involved in EG-VEGF/prokineticins-induced proliferation in BACE.

LeCouter and colleagues [8] showed that gene transfer of EG-VEGF in the rat ovary using adenovirus vector resulted in cysts formation with excessive angiogenesis. Future studies are required to examine the involvement of the EG-VEGF/prokineticin system in human ovarian disorders characterized by excessive angiogenesis, such as the polycystic ovary syndrome and ovarian cancer. The identification of EG-VEGF/prokineticins receptors in the ovarian capillary endothelial cells and elucidating the mechanism of EG-VEGF/prokineticins production in the ovary will be essential steps to clarify the pathological role of the EG-VEGF/prokineticins system in such human ovarian disorders. Furthermore, the wide tissue distributions of EG-VEGF/prokineticins and their receptors suggest that the EG-VEGF/prokineticin system, which plays a role in angiogenesis in endocrine glands and gastrointestinal motility, may also be involved in a number of additional, but as yet undefined, physiological roles.

In conclusion, we have demonstrated that EG-VEGF/prokineticin 1 and prokineticin 2 bind to and activate two structurally related GPCRs, EG-VEGF/PK-R1, and -R2. We have also shown the presence of both EG-VEGF/PK-R1 and -R2 mRNAs in BACE. The identification and characterization of these two distinct EG-VEGF/prokineticin receptors will facilitate the understanding of the physiological and/or pathological significance of EG-VEGF/prokineticin and may also lead to the development of a receptor antagonist that could be used to treat endocrine disorders involving excessive hypervascularity.

*Note added in proof.* During the preparation of the manuscript, a part of the study, the identification of human prokineticins/EG-VEGF receptors was reported [20].

## Acknowledgments

We thank Y. Matsumoto and H. Yoshida for helpful discussions.



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